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β-Xylosidase in strawberry fruit: Isolation of a full-length gene and analysis of its expression and enzymatic activity in cultivars with contrasting firmness

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Abstract

Strawberry is a non-climateric fleshy fruit, which softens quickly and has short post-harvest life. Ripening is associated with an increment of pectin solubility and a reduction of the content of hemicelluloses. In this work, we have cloned the full-length cDNA encoding a β-xylosidase (*FaXyl1*) from *Fragaria* × *ananassa* and we have characterized its expression in two strawberry cultivars with contrasting fruit firmness. The analysis of the predicted protein showed that *FaXyl1* is closely related to other β-xylosidases from higher plants. The recombinant protein obtained by over-expressing *FaXyl1* in *Escherichia coli* had β-xylosidase activity against the artificial substrate *p*-nitrophenyl β-D-xilopyranoside. Differently from other bifunctional xylosidases, no α-L-arabinofuranosidase activity was detected in the recombinant enzyme. The expression of *FaXyl1* gene was analyzed by northern-blot in Camarosa and Toyonaka strawberry cultivars, and compared with the corresponding protein data obtained by Western-blot and with the β-xylosidase activity during ripening. The softest cultivar (Toyonaka) showed an early accumulation of *FaXyl1* transcript and a higher expression of the corresponding protein during ripening, which correlates with a higher β-xylosidase activity in all ripening stages analyzed.

Keywords: Ripening; Softening; Cell wall; *Fragaria*

1. Introduction

Fruit ripening is known to be associated with characteristic changes of cell wall polysaccharides, which lead to the loosening of the structure and the softening of the flesh [1,2]. These changes would be mediated by the action of expansins [3] and hydrolases, the latter affecting mostly selective glycosidic bonds present in cell wall polysaccharides [4,5].

Strawberry is a non-climateric fruit showing a high softening rate, which contributes to its fast post-harvest decay. During strawberry fruit ripening, the total cell wall content decreases, while the percentage of water-soluble pectins increases, and this increment is higher in those cultivars with high softening rate [6,7]. It has been suggested that the increase in pectin solubility could be due to the cleavage of pectin side chains rather than pectin depolymerization [8]. First reports have showed that pectin depolymerization is slight [8], in accordance with the low polygalacturonase activity detected in strawberry [9]. However, in cultivars with fast softening, pectin depolymerization was detected during ripening, particularly in the covalently bound fraction [6]. Pectin metabolism could be also mediated by the action of pectate lyases [10]. Strawberry transgenic lines with antisense suppression of a putative pectate lyase gene showed a significant reduction of softening [10].

The content of hemicelluloses, one of the main components of cell wall, decreases during strawberry fruit ripening [7]. The activity of endoglucanase, a possible enzyme involved in hemicellulose degradation, increases during strawberry ripening [11], along with the expression of endo-β-1,4-glucanase genes

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[12,13]. However, strawberry transgenic lines with down-regulated expression of an endo-β-1,4-glucanase (cel1), showed no significant reduction of fruit softening [14]. Hemicelluloses mainly include xyloglucans and xylans. Xyloglucans are polymers of β-1,4-linked D-glucosyl residues substituted with α-linked D-xylosyl residue side groups and xylans consist of a backbone of β-1,4-linked D-xylosyl residues associated with side chains of 4-*O*-methylglucuronic acid and arabinose that are present in varying amounts in glucuronoxylans and arabinoxylans [15]. Xylan degradation occurs through the coordinated action of a variety of enzymes, including the endo-β-1,4-xylanases (EC 3.2.1.8), which cleave the β-1,4-glycosidic bonds between D-xylose residues in the main chain to produce xylooligosaccharides, and β-xylosidases (EC 3.2.1.37), which cleave xylooligosaccharides to release xylose [16].

While a number of β-xylosidases have been isolated from a variety of fungal and bacterial sources and their role in xylan hydrolysis is well documented [17–19], there is scarce information of these enzymes from higher plants. The activity of β-xylosidase enzyme has been reported in stone, avocado, olive, tomato, Japanese pear and strawberry fruits [20–25], but the corresponding β-xylosidase genes have been isolated only in the case of the three latter fruits. The objective of this study was to complete the cloning and characterization of *FaXyl1*, a β-xylosidase-like gene isolated from strawberry in our lab [25]. Particularly, we report the expression pattern of *FaXyl1*, the levels of the corresponding protein and the total β-xylosidase activity during ripening of two strawberry cultivars with contrasting fruit softening rates.

2. Materials and methods

2.1. Plant material

Strawberry fruits (*Fragaria* × *ananassa*, cv. Camarosa and Toyonaka) were obtained from local greenhouse producers. Fruits were harvested and classified according to their external color and size into different ripening stages: small green (SG), large green (LG), white (W), 50% red (50% R), 75% red (75% R) and 100% red (100% R). The peduncle and calyx were removed, and the fruit were immediately used or frozen in liquid nitrogen and stored at −80 °C.

2.2. 5′-End amplification of FaXyl1

A partial sequence, truncated at the 5′-end, of a putative β-xylosidase gene (*FaXyl1*) was isolated in a previous work [25]. The amplification of the 5′-end of *FaXyl1* was performed by RACE reaction from Toyonaka RNA (75% R) and the BD Smart™ RACE cDNA Amplification Kit (Clontech). Amplification of the 5′-end was performed using a reverse gene-specific primer GSP1 (5′-ATCGCTTCTGCCGCCGCTTCCTC-3′) and the Universal Primer A Mix (Clontech). BD Advantage 2 Polymerase Mix (Clontech) was used according to the manufacturer recommendations. The PCR product of the expected size was detected by electrophoresis on agarose gels, purified from the gel with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences), cloned into pGEM-T Easy Vector (Promega) and sequenced.

2.3. DNA sequencing and bioinformatics analysis

DNA was sequenced using Nested Universal Primer A (Clontech) and gene-specific primer GSP1. A sequencer Applied Biosystems ABI 377 was used (DNA Sequencing Service, Instituto de Investigaciones Biotecnológicas, UNSAM, Argentina). Sequence analyses were carried out using the Edit-Seq and Megalign programs included in the DNASTAR 4.05 software package. Glycosyl hydrolase domains were identified in the GeneBank database using the Conserved Domain Database with Reverse Position Specific Blast. Sub-cellular targeting and cleavage site of *FaXyl1*-deduced protein were performed using PSORT (http://psort.nibb.ac.jp/form.html) and SIGNALP (http://www.cbs.dtu.dk/services/SignalP) software. Potential *N*-glycosylation sites were studied using NetNGlyc software at http://www.cbs.dtu.dk/services/NetNGlyc/. Putative phylogenetic tree of deduced amino acid sequences was performed with the Megalign program, Clustal Method of the DNASTAR 4.05 software package, with a PAM250 residue weight table.

2.4. RNA isolation and northern blotting

Total RNA was isolated from frozen fruit using the hot borate method [26]. Each RNA sample (10 μg) was analyzed by electrophoresis in a 1.1% (w/v) agarose and 1% (v/v) formaldehyde denaturing gel. After running, RNA was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia), fixed by incubation for 2 h at 80 °C and cross-linked with a UV-Stratalinker Model 1800 (Stratagene). Membranes were prehybridized with 25 ml of hybridization solution at 42 °C for 4 h and then hybridized overnight at 42 °C with the denatured ^{32}P-labelled probe. The membranes were washed once at 42 °C and twice at 50 °C for 30 min each time in 25 ml of 1× SSC with 0.1% (w/v) SDS. The blot was exposed to X-ray film (X-OMAT AR, Kodak) with an intensifying screen at −80 °C, and the film was developed according to manufacturers' recommendation. Blots were hybridized with a probe for 18S rRNA to check that equal amounts of RNA had been loaded per lane.

2.5. Probe preparation

The β-xylosidase probe was prepared from the cDNA clone of *FaXyl1*. The restriction of this clone with *Eco*RI produced a fragment of approximately 800 bp that was purified and used as a template in a random priming labelling reaction using [^{32}P]dATP. To prepare the 18S rRNA template, a 200 bp fragment was amplified by PCR from a strawberry 18S rRNA clone (accession no. X15590) and the primers Rib5 (5′-ACCGTAGTAATTCTAGAGCT-3′) and Rib3 (5′-CCAC-TATCCTACCATCGAAA-3′). The probe was prepared using the PCR purified product as template in a random priming reaction in the presence of [^{32}P]dATP

2.6. Enzymatic activity assay

Frozen strawberries (10 g) were homogenized in an Omnimixer with 30 ml of the following extraction buffer: 0.05 M sodium acetate/acetic acid (pH 6.0), 1 M NaCl, 1% (w/v) PVPP. The mixture was left under stirring for 2 h and then centrifuged at 9000 × *g* for 30 min. The supernatant was used to determine β-xylosidase (EC 3.2.1.37) [16] and α-arabinofuranosidase (EC 3.2.1.55) activities.

To measure β-xylosidase activity, the following reaction mixture was prepared: 5 mM *p*-nitrophenyl β-D-xylopyranoside, 1 M NaCl, 0.05 M sodium acetate/acetic acid (pH 6.0), 750 μl of enzymatic extract in a total volume of 1500 μl. The mixture was incubated at 55 °C, aliquots of 150 μl were taken at different times and the reaction was stopped by adding 500 μl of 1% (w/v) Trizma base solution. In the control reactions, 750 μl of buffer 0.05 M sodium acetate/acetic acid (pH 6.0) plus 1 M NaCl was used instead of enzymatic extract.

The activity of α-arabinofuranosidase was measured from the following reaction mixture: 3 mM *p*-nitrophenyl-α-L-arabinofuranoside, 0.15 M sodium citrate/citric acid (pH 4.5), 250 μl of enzymatic extract in a total volume of 550 μl. The mixture was incubated at 37 °C, aliquots of 130 μl were taken at different times and the reaction was stopped by adding 150 μl 0.4 M Na_2CO_3 solution. In the control reactions, 250 μl of buffer 0.15 M sodium citrate/citric acid (pH 4.5) was used instead of enzymatic extract.

The amount of *p*-nitrophenol released in each reaction was determined by measuring the optical density at 410 nm and comparing with a calibration curve prepared from *p*-nitrophenol.

2.7. Heterologous expression

FaXyl1 truncated cDNA was amplified with primers XIL1 (5′-AAAGGATCCCACGAGTGGAAGCTC-3′) and XIL2 (5′-ACGCTCGAGCTGATCTAATTTCTC-3′). The amplified product was digested with *Bam*HI and *Xho*I and subcloned into pET-24a(+) vector (Novagen) digested with the same enzymes. The construct was transferred into *E. coli*, strain BL21 (DE3) (Stratagene). Transformed cells were cultured until OD_{600} = 0.5 and then induced with IPTG 1 mM at 37 °C for 3 h before harvesting. The bacteria culture was centrifuged at 8000 × *g* and both the pellet and the supernatant fractions were separated and saved. The former was used for purification of inclusion bodies and the latter was heat treated (see below) and then used to measure β-xylosidase activity. The same procedure was performed on cells transformed with the empty vector, and the absence of β-xylosidase activity was tested.

2.8. Heat treatment

The supernatant of bacteria lysate obtained after the induction with IPTG was incubated for 10, 20 and 30 min at 50, 55 and 60 °C in a water bath. After heat treatment, the mixture was clarified by centrifugation at 9000 × *g* for 20 min. The inactive precipitate was discarded and β-xylosidase activity was determined in the supernatant fraction. Three independent experiments of induction were performed and measurements of activity were done by duplicate for each time and incubation temperature. Data were expressed as media ± S.D.

2.9. FaXyl1 antisera production

The expression of *FaXyl1* in *E. coli* yielded insoluble protein aggregates in the form of inclusion bodies. The purification of these inclusion bodies was done according to Marston [27] and the protein isolated was used in an immunization protocol to produce antisera in rabbit [28].

2.10. Western blot

Frozen strawberries (3 g) were homogenized in an Omnimixer with 3 V of 50 mM Tris–HCl (pH 7.0), 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 1 mM EDTA, 5% (w/v) sucrose, and 1% (w/v) polyvinylpolypyrrolidone. The suspension was stirred for 40 min and centrifuged at 9000 × *g* for 30 min at 4 °C. The supernatant fraction was added with 0.1 V of 100% (w/v) trichloroacetic acid and incubated for 30 min at 4 °C. The suspension was centrifuged at 9000 × *g* for 5 min, the supernatant was discarded and the protein pellet was dissolved in 0.1 M NaCl and 1% (w/v) SDS.

Extracts containing 10 μg of proteins were separated by SDS-PAGE using 12% polyacrylamide gels [29] and electroblotted onto nitrocellulose membranes. Immunodetection was carried out with the ECL Western blotting analysis system (Amersham-Pharmacia) by using a 1:1000 dilution of the *Fragaria* × *ananassa* FaXyl1 antibody.

2.11. Preparation of native protein extracts and molecular exclusion chromatography

Toyonaka fruits (40 g) in 100% R ripening stage were homogenized in an Omnimixer with 3 V of 50 mM Tris–HCl (pH 7.0), 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 1 mM EDTA, 5% (w/v) sucrose, and 1% (w/v) polyvinylpolypyrrolidone. The suspension was stirred for 40 min, centrifuged at 9000 × *g* for 30 min and the supernatant fraction was concentrated by ammonium sulphate precipitation. Solid $(NH_4)_2SO_4$ was added slowly with gentle mixing to the supernatant fraction to bring the solution to 45% saturation. The solution was allowed to stand for 1 h and centrifuged at 9000 × *g* for 30 min. The precipitate was discarded and the supernatant fraction was brought to 85% saturation by adding solid ammonium sulphate and allowed to stand overnight at 4 °C. After centrifugation, the pellet was resuspended in buffer: 0.05 M sodium acetate/acetic acid (pH 6.0), 1 M NaCl. A volume of 1.6 ml of protein extract was fractionated on a Sephacryl S-100 (Amersham-Pharmacia) column (40 cm × 16 cm) pre-equilibrated in 0.05 M sodium acetate/acetic acid (pH 6.0), 1 M NaCl. The column was eluted with the same buffer at a flow rate of 0.4 ml min^{-1}. Fractions of 0.8 ml were collected and

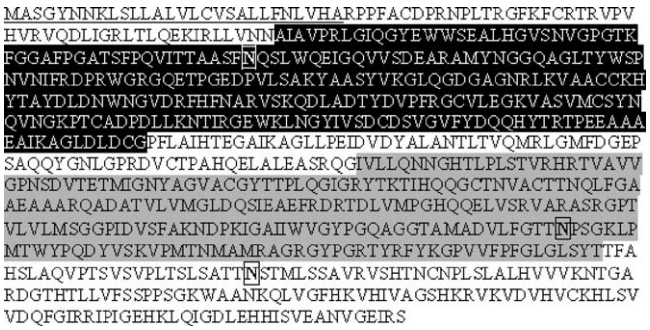

Fig. 1. Bioinformatic analysis of FaXyl1 protein. FaXyl1 amino acid sequence: underlined, signal peptide; black shading, glycosyl hydrolase family 3 N-terminal domain (gnl|CDD|25657 pfam00933); grey shading, glycosyl hydrolase family 3 C-terminal domain (gnl|CDD|25842 pfam01915); residues boxed in black, potential *N*-glycosylation sites.

0.425 ml of each fraction was utilized for assaying β-xylosidase activity.

2.12. Protein dosage

The extract protein concentration was measured by the modified Lowry method described by Potty [30], using bovine albumin as standard.

3. Results

3.1. Cloning of FaXyl1 full length

In a previous work, our group had isolated a clone from a strawberry cDNA library, which encodes for a putative β-xylosidase (*FaXyl1*), which was truncated in its 5′-end [25]. In this work, a 5′-RACE was performed using RNA from 75% red Toyonaka fruits, and we succeeded in obtaining the full-length cDNA of *FaXyl1*. The complete *FaXyl1* has an open reading frame of 2316 bp and a 3′-untranslated region of 214 bp. PSORT and SIGNALP software [31,32] predict a 28-amino acid signal peptide and the targeting of the cleaved product to the extracellular matrix. The predicted mature protein includes 744 amino acids; it has a molecular mass of 80.5 kDa and an isoelectric point of 8.29. According to the analysis of the sequence, the protein would posses the features corresponding to the glycosyl-hidrolase family. Conserved domains, which are present in glycosyl-hidrolase family 3, are present in the deduced amino acid sequence of *FaXyl1* (Fig. 1). Finally, FaXyl1 possesses three potential sites for *N*-glycosylation, according to its predicted subcellular localization.

Phylogenetic analysis of the deduced amino acid sequence of *FaXyl1* showed a high identity with sequences of β-xylosidases from higher plants and a clear divergence with sequences belonging to fungi and oomycetes (Fig. 2).

3.2. Expression of FaXyl1 during fruit ripening

Northern blot analysis was used to determine the expression of *FaXyl1* during fruit ripening of two strawberry cultivars with contrasting firmness (Fig. 3A). One of them, Toyonaka, softens fast and renders very soft fruit while the other (Camarosa) has a slower softening rate and the fruits are firmer. In Toyonaka fruits, mRNA levels for *FaXyl1* were low in small green fruit and increased in white fruit, reaching the maximum at 50% red stage and diminishing thereafter. On the contrary, transcript accumulation was very low in Camarosa, showing a slight increase late at the end of ripening (100% red stage).

3.3. Activity and stability of the recombinant enzyme

The overexpression of *FaXyl1* in *E. coli* yielded insoluble protein aggregates in the form of inclusion bodies. However, a portion of the recombinant enzyme remained in the soluble fraction and had β-xylosidase activity on *p*-nitrophenyl β-D-xilopyranoside as substrate. β-Xylosidases isolated from different plant sources showed a moderate resistance to high temperatures [33,34]. The thermal stability of the recombinant FaXyl1 was evaluated by measuring the remaining activity after incubating the bacteria lysate supernatant at temperatures from 50 to 60 °C for 10 to 30 min. After the treatment, the mixture was clarified by centrifugation and the β-xylosidase activity measured. β-Xylosidase activity was almost unaffected by

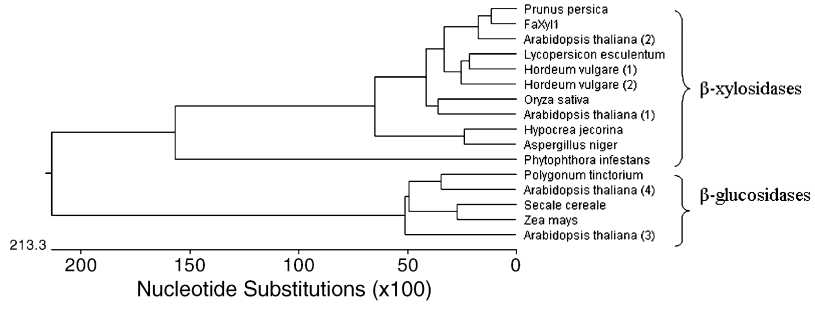

Fig. 2. Phylogenetic tree of deduced amino acid sequences of β-xylosidases and β-glucosidases. The tree was constructed using the MegAlign program, Clustal method, with a PAM250 residue weight table. The GenBank accession numbers of the sequences are: β-xylosidases: *Arabidopsis thaliana* (1), AAF17692; *Aspergillus niger*, AF108944; *Fragaria* × *ananassa* (FaXyl1), AAS17751; *Hordeum vulgare* (1), AYO29260; *Hypocrea jecorina*, CAA93248; *Lycopersicon esculentum*, AB041811; *Oryza sativa*, BAB55751; *Phytophthora infestans*, AF352032; *Prunus persica*, ACO22521. α-Arabinofuranosidase/β-xylosidases: *Arabidopsis thaliana* (2), BAB09906; *Hordeum vulgare* (2), AAK38481. β-Glucosidases: *Arabidopsis thaliana* (3), NM179517; *Arabidopsis thaliana* (4), AK117809; *Polygonum tinctorium*, ABO03039; *Secale cereale*, AF293849; *Zea mays*, U33816.

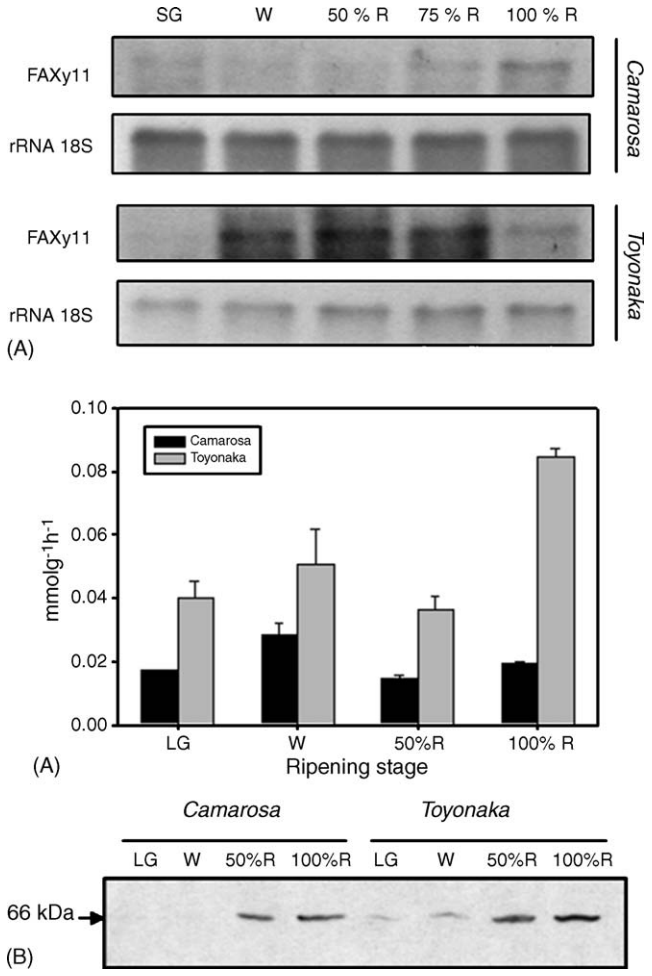

Fig. 3. Characterization of *FaXyl1* during strawberry fruit ripening. (A) Northern hybridization analysis of *FaXyl1* expression. Total RNA from small green (SG), white (W), 50% red (50% R), 75% red (75% R), and 100% red (100% R) fruits was electrophoresed and then hybridized with *FaXyl1* radiolabelled probe. rRNA 18S was used as a control for RNA loading. (B) Change of β-xylosidase activity. Protein extracts were prepared from large green (LG), white (W), 50% red (50% R), and 100% red (100% R) fruits. Bars indicate standard deviations. (C) Western blot analysis of FaXyl1 protein. Antibodies raised against FaXyl1 truncated protein were used to probe a Western blot of protein extracts prepared from large green (LG), white (W), 50% red (50% R), and 100% red (100% R) fruits.

incubations at 50 or 55 °C, for 30 or 20 min, respectively. However, as protein precipitates with higher temperatures and longer incubations, there was a net increase of specific activity (Table 1). The incubation at 55 °C for 30 min enhanced the protein precipitation but also reduced the activity with a reduction of the specific activity. Differently, incubation at 60 °C highly reduced the activity even after 10 min of incubation and provoked a low specific activity. According to these results, β-xylosidase activity was determined at 55 °C in subsequent experiments.

Most β-xylosidases are bifunctional and also have α-L-arabinofuranosidase activity [35,36]. Both enzyme activities were measured in the FaXyl1 recombinant enzyme and in extracts obtained from fruit of Toyonaka cultivar at 100% R stage (Table 2). Strawberry fruit showed both β-xylosidase and

Table 1
Heat treatment of FaXyl1 recombinant protein

Incubation		Activity (nmol min^{-1} ml^{-1})	Protein (mg ml^{-1})	Specific activity (nmol min^{-1} mg^{-1})
Temperature (°C)	Time (min)			
50	10	0.499 ± 0.031	5.8	0.086 ± 0.005
	20	0.506 ± 0.003	4.6	0.110 ± 0.006
	30	0.508 ± 0.007	4.1	0.124 ± 0.002
55	10	0.551 ± 0.011	4.4	0.125 ± 0.003
	20	0.543 ± 0.003	3.5	0.155 ± 0.001
	30	0.446 ± 0.006	3.0	0.149 ± 0.002
60	10	0.078 ± 0.005	2.8	0.028 ± 0.002
	20	0.033 ± 0.002	2.8	0.012 ± 0.001
	30	0.000 ± 0.000	2.3	0.000 ± 0.000

The supernatant of bacteria lysate was incubated at several combinations of time-temperature and the remained β-xylosidase activity was determined.

α-L-arabinofuranosidase activities. However, FaXyl1 recombinant protein presented only β-xylosidase activity. No liberation of *p*-nitrophenol was detected when *p*-nitrophenol-α-L-arabinofuranoside was utilized as substrate.

3.4. β-Xylosidase activity during fruit ripening

β-Xylosidase activity was measured during fruit ripening in both cultivars. The total β-xylosidase enzyme activity was detected in all the ripening stages analyzed and was higher in Toyonaka cultivar (Fig. 3B). In both cultivars, the activity increased from LG to W stage and then decreased to 50% R. Afterwards, the activity increased again in 100% R fruits. The latter increase was slight in Camarosa but very high in Toyonaka fruits. Therefore, the highest β-xylosidase activity was found in W and 100% R stages in Camarosa and Toyonaka fruits, respectively.

3.5. Western blot analysis

The over-expression of *FaXyl1* in *E. coli* allowed obtaining the protein in inclusion bodies that were purified for the subsequent production of the β-xylosidase antisera in rabbit. Antibodies produced against *FaXyl1* were used in a Western blot analysis of total protein extracts obtained from fruits of Camarosa and Toyonaka cultivars. The presence of a single band of molecular mass 66 kDa was detected in both cultivars (Fig. 3C). In Toyonaka fruits, FaXyl1 was present in all stages

Table 2
Measurements of β-xylosidase and α-arabinofuranosidase activities in extracts obtained from fruits of Toyonaka cultivar at 100% R and *E. coli* cells transformed with *FaXyl1*

Enzyme source	β-Xylosidase specific activity (nmol min^{-1} mg^{-1})	α-Arabinofuranosidase specific activity (nmol min^{-1} mg^{-1})
Toyonaka 100% R	0.523 ± 0.008	0.726 ± 0.027
FaXyl1 recombinant protein	0.152 ± 0.011	Not detected

analyzed and its amount increased during ripening. Otherwise, FaXyl1 protein was not detected until 50% red stage in Camarosa and only a slight increase was observed at 100% stage.

3.6. Molecular exclusion chromatography

Protein extracts obtained from unripe and ripe fruits of both cultivars were analyzed by exclusion chromatography to investigate the presence of β-xylosidase isozymes. No β-xylosidase activity could be detected in the chromatographic fractions of extracts obtained neither from Camarosa nor from unripe Toyonaka fruits, probably due to the low initial activity of the extracts and the dilution occurring during column elution. However, a single peak of β-xylosidase activity was found using extracts from 100% red Toyonaka fruits, which corresponds to an apparent molecular mass of 66 kDa (data not shown).

4. Discussion and conclusion

The *FaXyl1* clone isolated previously by Martínez et al. [25] had a size of 1699 bp and was truncated in its 5′-end. The RACE experiments described in this work allowed cloning the 5′-end and obtaining the full-length cDNA of *FaXyl1*. Protein encoded by *FaXyl1* belongs to the family 3 of glycoside hydrolases and shows high identity with sequences of β-xylosidases from higher plants particularly with the xylosidase sequences reported in peach fruit [37]. In the phylogenetic analysis we also included several β-glucosidases, since the family 3 of glycosil hydrolases is formed not only by β-xylosidases but also by β-glucosidases. Clearly, *FaXyl1* has higher proximity to other β-xylosidases, rather than to the divergent group of β-glucosidases (Fig. 2). The protein encoded by *FaXyl1* contains a predicted signal peptide and seems to be targeted to the extracellular matrix. The presence of several potential *N*-glycosylation sites in the amino acid sequence is in accordance with the extracellular location. β-Xylosidases are enzymes commonly found in the stock of cell wall degrading enzymes of pathogens such as bacteria and fungi and could be active toward cell wall components [38]. However, several studies have reported their presence in plants [16,20–25,37], and the participation of β-xylosidases in hemicellulose metabolism during plant development has been proposed in *A. thaliana* [39]. The full-length cDNA encodes a primary translation product of 772 amino acid residues, with a predicted molecular mass of 81 kDa. This value is higher than the apparent molecular mass value of 66 kDa found by molecular exclusion and Western-blot analysis. Similar results were obtained in β-xylosidases purified from both stems of *A. thaliana* and seedlings of *Hordeum vulgare* [35,36]. In the latter case, the authors proved the processing of COOH-terminal portion of the primary translation product during maturation. Probably, in strawberry fruit, such a deletion could also occur, causing the reduction of molecular mass from 81 kDa (predicted from cDNA) to 66 kDa (Western blot and chromatography).

The cDNA of *FaXyl1* was used to obtain the recombinant protein expressed *in vitro* in *E. coli*. A high amount of protein was stored in the inclusion bodies, but some of the recombinant enzyme remained soluble. The enzyme activity measured in the soluble fraction confirmed that the isolated *FaXyl1* clone encodes effectively for a β-xylosidase. As other members of this group, the enzyme obtained from strawberry showed a relatively high thermal stability [33,34], which allowed determining the activity at high temperatures, and this feature could facilitate the enzyme purification by introducing a heating step to eliminate interfering proteins.

β-Xylosidases have activity toward artificial and natural substrates containing D-xylose. However, in many cases, they also hydrolyze "*in vitro*" *p*-nitrophenyl-α-L-arabinofuranoside, suggesting a possible arabinofuranosidase activity. In *Arabidopsis*, it was shown that XYL1 efficiently hydrolyze L-arabinofuranose indicating that the enzyme is bifunctional [35]. However, XYL4, also obtained from *Arabidopsis*, possesses an enzymatic specificity characteristic for a β-xylosidase with low activity toward *p*-nitrophenyl-α-L-arabinofuranoside [35]. In barley, a β-xylosidase (XYL) purified from seedlings showed a very low α-arabinofuranosidase activity. Differently, the corresponding α-arabinofuranosidase (ARA-I) has bifunctional (β-xylosidase and α-arabinofuranosidase) catalytic properties [36]. In our case, strawberry fruit effectively showed α-arabinofuranosidase activity but the recombinant enzyme was active only toward the artificial substrate *p*-nitrophenyl β-D-xylopyranoside. These results indicate that FaXyl1 does not have "*in vitro*" α-arabinofuranosidase activity toward artificial substrates and that the activity detected in fruits was due to an enzyme different that FaXyl1. However, the possibility that FaXyl1 could "*in vivo*" liberate α-L-arabinofuranose residues from cell wall polymers should not be discarded.

Previous works that analyzed the ripening of strawberry fruits proved the presence of a range of hydrolytic enzymes, such as polygalacturonase [40], β-galactosidase [41], endo-β-1,4-glucanase [42–45], pectin methylesterase [46] and β-xylosidase [25]. In this work, we have characterized the changes of β-xylosidase activity during ripening of two strawberry varieties with contrasting fruit softening rate. Camarosa cultivar produces fruits with higher firmness values than Toyonaka cultivar at all ripening stages [6]. The difference in firmness between both cultivars is such that Camarosa fruits at 100% R have similar firmness than Toyonaka fruits at 25% R stage [6]. According to the data shown in the present work, there is a clear correlation between *FaXyl1* expression or β-xylosidase activity and the fruit softness. The expression of *FaXyl1* was higher in Toyonaka, the softest cultivar, than in Camarosa. The analysis by Western-blot detected FaXyl1 protein in all ripening stages and in higher quantities in the softest cultivar, while in Camarosa the protein was detected only from 50% red stage.

However, a correlation between the β-xylosidase activity and the expression of *FaXyl1* or the corresponding protein in different ripening stages is not clear. In Toyonaka, the maximum mRNA content was found in 50% red stage while the activity and enzyme amount were highest at the end of

ripening. A similar pattern was observed in the expression of *FaExp*2, a gene encoding a ripening related expansin, which started its expression in W stage while the corresponding protein was detected by Western blot at the Turning stage [47]. In Camarosa, Northern and Western blot analyses correlated, showing the highest expression of both mRNA and protein at 100% red stage, but maximum β-xylosidase activity was found at the W stage. A similar inconsistency between enzyme activity and expression pattern of β-xylosidase encoding genes was also described in tomato fruit [23]. Differences in the patterns of activity and mRNA expression could be due to the activity of other proteins with β-xylosidase activity. Although only one peak with enzyme activity was detected by molecular exclusion chromatography, it should not be discarded the presence of several isoenzymes of similar molecular mass or isoenzymes with a molecular mass different of 66 kDa and low activity, which became undetectable by chromatography analysis after the different steps of extraction and purification. An additional complexity in the expression pattern was found when a comparison among strawberry cultivars was performed. The enzyme activity and mRNA patterns during ripening of cultivars analyzed in the present work are different from those described in Selva cultivar [25]. Moreover, differences in cell wall composition were detected during ripening of cultivars with different softening rate [6]. Both facts indicate that the physiology and metabolism of cell wall could vary significantly among strawberry cultivars with contrasting fruit firmness.

The variation of β-xylosidase activity or the expression of putative genes encoding β-xylosidases does not follow the same pattern in all the fruits analyzed up to date. During ripening, β-xylosidase activity increases in Japanese pear [24], decreases in tomato [23] and increases and then decreases in avocado fruit [21]. Regarding the expression of putative β-xylosidase genes, in tomato the expression of *LeXyl1* increases while that of *LeXyl2* decreases during ripening [23]. In pear and peach, the highest accumulation of β-xylosidase mRNA was found at the end of ripening [24,37]. In the case of strawberry, the pattern of enzyme activity differs with the cultivar analyzed: β-xylosidase activity peaked at 50% R in Selva [25] and at W in Camarosa and Toyonaka, and the activity increased notoriously at 100% R in the latter cultivar (this work). The expression of *FaXyl1* is also cultivar dependent (this work and Ref. [25]).

Altogether, data from different fruit suggest that the biochemical role of β-xylosidase would be accomplished at different developmental or ripening stages in different fruits. The expression of different cell wall modifying proteins at different stages is a very common pattern in fruit [48], which suggests that prior action of some of the enzymes is necessary before others can act on a wall-localized polymer substrate or that different isoforms of a given enzyme play distinct roles at different developmental stages.

Strawberry fruit ripening is associated with a significant reduction in the content of hemicelluloses [6,49]. Several endoglucanase genes express specifically in fruit and at high level in ripe strawberries [42–45], which supports their probable role in xyloglucan degradation. In addition to xyloglucans, dicots have small amounts of other hemicellulosic polymers, including β-1,4-xylans. Although the amount of xylans has not been directly determined in the strawberry fruit cell wall, a high xylose:glucose ratio in the hemicellulosic fraction has been reported [7,8,49], suggesting the presence of Xyl-containing polymers. In strawberry cell walls, the amount of xylose is higher in the hemicellulose fraction than in the pectin fraction [49], but the presence of xylogalacturonans should not be discarded. In apple fruit (another member of Rosaceae), Schols et al. [50] have demonstrated the presence of these compounds and reported that single β-xylosyl residues could be released from the xylogalacturonan backbone by treatment with a fungal β-xylosidase [50]. These data suggest that both xylan and xylogalacturonan could be targets for β-xylosidase action.

As a conclusion, we could establish a higher β-xylosidase activity and a higher *FaXyl1* mRNA and FaXyl1 protein accumulation in the softest strawberry cultivar. Moreover, protein encoded by *FaXyl1* clearly showed β-xylosidase activity against artificial substrates, proving that the product of this gene is the mentioned enzyme. Although these results suggest a possible role of *FaXyl1* in fruit softening, additional works are required to establish the *in vivo* action of the enzyme.

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References

[1] P.M. Dey, K. Brinson, Plant cell walls, Adv. Carbohydr. Chem. Biochem. 42 (1984) 265–382.
[2] K.C. Gross, C.E. Sams, Changes in cell wall neutral sugar composition during fruit ripening: a species survey, Phytochemistry 23 (1984) 2457–2461.
[3] J. Rose, H. Lee, A. Bennett, Expression of a divergent expansin gene is fruit-specific and ripening-regulated, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 5955–5960.
[4] D.J. Huber, The role of cell wall hydrolases in fruit softening, Hortic. Rev. 5 (1983) 169–219.
[5] C.J. Brady, Fruit ripening, Annu. Rev. Plant Physiol. 38 (1987) 155–178.
[6] H.G. Rosli, P.M. Civello, G.A. Martínez, Changes in cell wall composition of three *Fragaria* × *ananassa* cultivars with different softening rate during ripening, Plant Physiol. Biochem. 42 (2004) 823–831.
[7] D.J. Huber, Strawberry fruit softening: the potential roles of polyuronides and hemicelluloses, J. Food Sci. 49 (1984) 1310–1315.
[8] Y. Nogata, K. Yoza, K. Kusumoto, H. Ohta, Changes in molecular weight and carbohydrate composition of cell wall polyuronide and hemicellulose during ripening in strawberry fruit, in: J. Visser, A.G.J. Voragen (Eds.), Pectins and Pectinases, Elsevier Science, Amsterdam, 1996, pp. 591–596.
[9] Y. Nogata, H. Ohta, A.G.J. Voragen, Polygalacturonase in strawberry fruit, Phytochemistry 34 (1993) 617–620.
[10] S. Jiménez-Bermúdez, J. Redondo-Nevado, J. Muñoz-Blanco, J.L. Caballero, J.M. López-Aranda, V. Valpuesta, F. Pliego-Alfaro, M.A. Quesada, J.A. Mercado, Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene, Plant Physiol. 128 (2002) 751–759.
[11] F.B. Abeles, F. Takeda, Cellulase activity and ethylene in ripening strawberry and apple fruit, Sci. Hortic. 42 (1990) 269–275.
[12] M.H. Harpster, D.A. Brummell, P. Dunsmuir, Expression analysis of a ripening-specific, auxin-repressed endo-1,4-beta-glucanase gene in strawberry (*Fragaria* × *ananassa*), Plant Physiol. 118 (1998) 1307–1316.

[13] I. Llop-Tous, E. Domínguez-Puigjaner, X. Palomer, M. Vendrell, Characterization of two divergent endo-β-1,4-glucanase cDNA clones highly expressed in the nonclimacteric strawberry fruit, Plant Physiol. 119 (1999) 1415–1421.
[14] L.C. Woolley, D.J. James, K. Manning, Purification and properties of an endo-β-1,4-glucanase from strawberry and down-regulation of the corresponding gene, *cel1*, Planta 214 (2001) 11–21.
[15] N. Carpita, M. McCann, The cell wall, in: B.B. Buchanan, W. Gruissem, R.L. Jones (Eds.), Biochemistry and Molecular Biology of Plants, American Society of Plant Physiologists, Rockville, MD, 2000, pp. 52–108.
[16] G. Cleemput, M. Hessing, M. van Oort, M. Deconynck, J.A. de Cour, Purification and characterization of a β-xylosidase and an endoxylanase from wheat flour, Plant Physiol. 113 (1997) 377–386.
[17] E. Margolles-Clark, E.M. Tenkanen, T. Nakari-Setala, M. Penttila, Cloning of genes encoding α-L-arabinofuranosidase and β-D-xylosidase from *Trichoderma reisei* by expression in *Saccharomyces cerevisiae*, Appl. Environ. Microbiol. 62 (1996) 3840–3846.
[18] J.A. Pérez-González, N.N. van Peij, A. Bezoen, A.P. MacCabe, D. Ramon, L.H. deGras, Molecular cloning and transcriptional regulation of the *Aspergillus nidulans* xlnD gene encoding a betaxylosidase, Appl. Environ. Microbiol. 64 (1998) 1412–1419.
[19] N.N. van Peij, J. Brinkmann, M. Vrsanská, J. Visser, L. de Graaf, β-Xylosidase activity, encoded by xlnD, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of xylanolitic enzyme spectrum, Eur. J. Biochem. 245 (1997) 164–173.
[20] D.L. Bouranis, C.A. Niavis, Cell wall metabolism in growing and ripening stone fruits, Plant Cell Physiol. 33 (1992) 999–1008.
[21] R. Ronen, G. Zauberman, M. Akerman, A. Weksler, I. Rot, Y. Fuchs, Xylanase, xylosidase activities in avocado fruit, Plant Physiol. 95 (1991) 961–964.
[22] J.F. Bolaños, R. Rodríguez, R. Guillén, A. Jiménez, A. Heredia, Activity of cell wall-associated enzymes in ripening olive fruit, Physiol. Plant. 93 (1995) 651–658.
[23] A. Itai, K. Ishihara, D. Bewley, Characterization of expression, and cloning, of β-D-xylosidase and α-L-arabinofuranosidase in developing and ripening tomato (*Lycopersicon esculentum* Mill.) fruit, J. Exp. Bot. 54 (2003) 2615–2622.
[24] A. Itai, K. Yoshida, K. Tanabe, F. Tamura, A β-D-xylosidase-like gene is expressed during fruit ripening in Japanese pear (*Pyrus pyrifolia Nakai*), J. Exp. Bot. 50 (1999) 877–878.
[25] G.A. Martínez, A.R. Chávez, P.M. Civello, β-xylosidase activity and expression of a β-xylosidase gene during strawberry fruit ripening, Plant Physiol. Biochem. 42 (2004) 89–96.
[26] C. Wan, T.A. Wilkins, A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.), Anal. Biochem. 223 (1994) 7–12.
[27] F.A.O. Marston, The purification of eukaryotic polypeptides synthesized in *E. coli*, Biochem. J. 240 (1986) 1–12.
[28] D. Catty, Antibodies: A Practical Approach, vol. 1, IRL Press, Oxford, 1988.
[29] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[30] V.H. Potty, Determination of protein in the presence of phenols and pectins, Anal. Biochem. 29 (1969) 535–539.
[31] K. Nakai, P. Horton, PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization, Trends Biochem. Sci. 24 (1999) 34–36.
[32] H. Nielsen, J. Engelbrecht, S. Brunak, G. von Heijne, A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites, Int. J. Neural Syst. 8 (1997) 581–599.
[33] C.V. Mujer, A.R. Miller, Purification and properties of β-xylosidase isozymes from cucumber seeds, Physiol. Plant. 82 (1991) 367–376.
[34] I. Chinen, K. Oouchi, H. Tamaki, N. Fukuda, Purification and properties of thermoestable β-xylosidase from immature stalks of *Saccharum officinarum* L. (sugar cane), J. Biochem. 92 (1982) 1873–1881.
[35] Z. Minic, C. Rihouey, C. Trung Do, P. Lerouge, L. Jouanin, Purification and characterization of enzymes exhibiting β-D-xylosidase activities in stem tissues of *Arabidopsis*, Plant Physiol. 135 (2004) 1–12.
[36] R.C. Lee, M. Hrmova, R.A. Burton, J. Lahnstein, G.B. Fincher, Bifunctional family 3 glycoside hydrolases from barley with α-L-arabinofuranosidase and β-D-xylosidase activity, J. Biol. Chem. 278 (2003) 5377–5387.
[37] B. Ruperti, L. Cattivelli, S. Pagni, A. Ramina, Ethylene-responsive genes are differentially regulated during abscission, organ senescence and wounding in peach (*Prunus persica*), J. Exp. Bot. 53 (2002) 429–437.
[38] R.P. de Vries, H.C. Kester, C.H. Poulsen, J.A. Benen, J. Visser, Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides, Carbohydrates 327 (2000) 860–865.
[39] T. Goujon, Z. Minic, A. El Amrani, O. Lerouxel, E. Aletti, C. Lapierre, J. Joselau, L. Jouanin, AtBXL1, a novel higher plant (*Arabidopsis thaliana*) putative beta-xylosidase gene, is involved in secondary cell wall metabolism and plant development, Plant J. 33 (2003) 677–690.
[40] Y. Nogata, H. Ohta, A.G.J. Voragen, Polygalacturonase in strawberry fruit, Phytochemistry 34 (1993) 617–620.
[41] L. Trainotti, R. Spinello, A. Piovan, S. Spolaore, G. Casadoro, β-Galactosidases with a lectin-like domain are expressed in strawberry, J. Exp. Bot. 52 (2001) 1635–1645.
[42] M.H. Harpster, D.A. Brummell, P. Dunsmuir, Expression analysis of a ripening-specific, auxin-repressed endo-1,4-β-glucanase gene in strawberry, Plant Physiol. 118 (1998) 1307–1316.
[43] I. Llop-Tous, E. Dominguez-Puigjaner, X. Palomer, M. Vendrell, Characterization of two divergent endo-1,4-β-glucanase cDNA clones highly expressed in the nonclimateric strawberry fruit, Plant Physiol. 119 (1999) 1415–1421.
[44] K. Manning, Isolation of a set of ripening-related genes from strawberry: their identification and possible relationship to fruit quality traits, Planta 205 (1998) 622–631.
[45] L. Trainotti, S. Spolaore, A. Pavanello, B. Baldan, G. Casadoro, A novel E-type endo-1,4-β-glucanase with a putative cellulose-binding domain is highly expressed in ripening strawberry fruits, Plant Mol. Biol. 40 (1999) 323–332.
[46] G.E. Neal, Changes occurring in the cell walls of strawberries during ripening, J. Sci. Food Agric. 16 (1965) 604–611.
[47] P.M. Civello, A. Powell, A.B. Bennett, Expression of a ripening-regulated expansin gene in strawberry, Plant Physiol. 121 (1999) 1273–1279.
[48] D.A. Brummell, M.H. Harpster, Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants, Plant Mol. Biol. 47 (2001) 311–340.
[49] T. Kohn, L. Melton, Ripening-related changes in cell wall polysaccharides of strawberry cortical and pith tissues, Post. Biol. Technol. 26 (2002) 23–33.
[50] H. Schols, E. Vierhuis, E. Bakx, A. Voragen, Different populations of pectic hairy regions occur in apple cell walls, Carbohydr. Res. 275 (1995) 343–360.